Primary Ovarian Carcinomas Display Multiple Methylator Phenotypes Involving Known Tumor Suppressor Genes

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Mounting evidence suggests that aberrant methylation of CpG islands is a major pathway leading to the inactivation of tumor suppressor genes and the development of cancer. Recent studies on colorectal and gastric cancer have defined a CpG island methylator phenotype (CIMP), which involves the targeting of multiple genes by promoter hypermethylation. To determine the role of methylation in ovarian cancer, we have investigated the methylation status of 93 primary ovarian tumors at ten loci using methylation-specific polymerase chain reaction (MSP). Seven of the loci (*BRCA1*, *HIC1*, *MINT25*, *MINT31*, *MLH1*, *p73* and *bTR*) were found to be methylated in a significant proportion of the ovarian tumors, and methylation of at least one of these was found in the majority (71%) of samples. Although concurrent methylation of multiple genes was commonly seen, this did not seem to be due to a single CIMP phenotype. Instead the results suggest the presence of at least three groups of tumors, two CIMP-positive groups, each susceptible to methylation of a different subset of genes, and a further group of tumors not susceptible to CpG island methylation, at least at the loci studied. (*Am J Pathol* 2001, 158:1121–1127)

In human genomic DNA the CpG dinucleotide is generally underrepresented and the majority exhibit methylation at the 5' position of the cytosine.¹ However, about 60% of human genes are associated with CpG islands, which are comparatively rich in CpG dinucleotides and are almost always methylation free in adult tissues.¹ In cancer the methylation of DNA is frequently dysregulated, leading to both genome-wide hypomethylation and local areas of hypermethylation, usually in the CpG islands

associated with tumor suppressor genes.² Recently, the importance of the role of aberrant methylation in the development of cancer has become increasingly apparent with a growing list of genes, such as *p16*,³ *Rb*,⁴ and *BRCA1*,⁵ shown to be susceptible to inactivation by promoter hypermethylation. Indeed in some cases, such as loss of expression of the mismatch repair gene *MLH1*, promoter hypermethylation appears to represent the predominant mechanism by which expression of this gene is lost in tumors.^{6–8}

A number of recent advances in the methodology for investigating DNA methylation have greatly facilitated the analysis of the role of methylation in cancer. In particular, polymerase chain reaction (PCR)-based techniques involving sodium bisulfite modification of DNA^{9,10} have allowed much more rapid analysis of large tumor sets than was previously possible. Using these techniques, two studies of colorectal¹¹ and gastric cancer¹² have demonstrated that methylation dependent gene inactivation is not randomly distributed but that a subset of tumors display a methylator phenotype, termed CpG island methylator phenotype (CIMP), in which multiple genes are concurrently methylated. These results were largely based on a series of recently identified genes known as MINTs (*methylated in tumors*),¹³ but also involved genes known to play important roles in tumor development, such as *p16* and *MLH1*. Concurrent methylation of multiple genes has also been demonstrated in acute myeloid leukemia.¹⁴

Little is currently known about the role of methylation in ovarian cancer. The majority of studies to date have focused on the *p16* gene, but although loss of *p16* expression is seen in a proportion of ovarian tumors, conflicting results as to whether or not methylation plays a role in this loss of expression have been obtained.^{15,16} Three reports have demonstrated a role for methylation in inactivation of the familial cancer gene *BRCA1*, with between 5% and 13% of tumors displaying methylation^{17–19} and methylation in a chromosomal region known to contain the putative tumor suppressor gene *HIC1* has been

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reported in 33% of ovarian cancers.²⁰ We have also recently described *MLH1* methylation in ovarian cancer and its role in chemotherapeutic drug resistance.²¹ In addition, the recently cloned *hTR* promoter²² has been shown to be methylated in some ovarian tumors (N. Keith, personal communication). To more clearly determine the role of aberrant methylation in ovarian cancer we have studied a large series of ovarian tumors at multiple loci using methylation-specific PCR (MSP). We now report that seven of the loci studied (*MLH1*, *HIC1*, *hTR*, *BRCA1*, *p73*, *MINT25*, and *MINT31*) exhibited promoter hypermethylation in a significant proportion of the samples. However, unlike the previous reports in colorectal and gastric cancer, we find that not all methylated loci are coordinately methylated. Instead, the results suggest there may be multiple methylator phenotypes in ovarian cancer.

Materials and Methods

Tissue Samples

Ovarian tumor and normal samples were obtained from the Western Infirmary and Stobhill General hospitals (Glasgow, UK) and Pembury Hospital (Kent, UK). The samples were stored frozen at -70°C . Pathology reports, including grade and histological subtype, were obtained where possible.

Genomic DNA was extracted for methylation analysis as previously described.²¹

MSP

MSP was performed largely as described before.⁹ Between 250 μg and 1 mg of genomic DNA was modified with sodium bisulfite using the CpGenome modification kit (Intergen, Purchase, NY) per the manufacturer's instructions. All samples were resuspended in 40 μl of TE, and 1 μl of this was used for subsequent PCR reactions. Correct modification was confirmed using amplification with primers specific for unmethylated DNA. Subsequently the samples were amplified with primers specific for methylated DNA at the ten loci being analyzed. Three primer sets (*hTR*, *p16U*, *P16M*) were amplified in 25- μl volumes containing 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1–2 mmol/L MgCl_2 , 10 mmol/L dNTPs, 0.75 units of taq polymerase (Roche, Lewes, UK) and 75 ng of each primer. All other primers were amplified in 25 μl reactions containing 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1.5–4 mmol/L MgCl_2 , 10 mmol/L dNTPs, 1.25 units of AmpliTaq Gold polymerase (Perkin Elmer, Branchburg, NJ) and 75 ng of each primer. PCR was performed with one cycle of 95°C for 5 minutes (Taq polymerase) or 12 minutes (AmpliTaq Gold), 35 cycles of 95°C for 30 seconds, $55\text{--}63^{\circ}\text{C}$ for 30 seconds, and 72°C for 30 seconds, followed by one cycle of 72°C for 5 minutes. All PCR reactions were carried out on a Touch-down thermocycler (Hybaid, Ashford, UK). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. Samples giving signals ap-

proximately equivalent to the positive control were designated as methylated. Samples giving faint positive signals were repeated on multiple occasions and only those that were consistently positive were designated as methylated. The primers used for the PCR reactions were as follows: *BRCA1*, forward 5'-GAGTTTCGAGAGACGTT-TGG-3', reverse 5'-AATCTCAACGAACTCACGCC-3'; *hTR*, forward 5'-GACGTAAAGTTTTTTCGGACG-3', reverse 5'-ACCCGAT ACGCTACCGAACG-3'; *HIC1*, forward 5'-TTCGGGTTAGGGTCGTAGTC-3', reverse 5'-CTAACCG-AAAACAT CAACCCCTCG-3'; *MINT25*, forward 5'- GCGA AAGCGAAAGTCGTTTCG-3', reverse 5'- CCGAACGCACATA-ACGAACC-3'; *MINT31*, forward 5'-AGGGTAATTAGG-GAGACGAC-3', reverse 5'- AAAACGCTTACGCCACTACG-3'; *p73*, forward 5'-GTTTCGCGTTGTTTTTCGCG-3', reverse 5'-AATACCTACCCAACGCTACG-3'. The sequences of the other primers used have been previously reported.^{6,9,23}

Immunohistochemistry

Immunohistochemistry was carried out on paraffin-embedded tumor samples as described before²⁴ using a mouse anti-MLH1 antibody (Pharmingen, San Diego, CA) at a concentration of 2.5 $\mu\text{g}/\text{ml}$ or a mouse anti-BRCA1 (Calbiochem, La Jolla, CA) at a concentration of 0.67 $\mu\text{g}/\text{ml}$. Detection was carried out using a Vectastain ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Both the intensity of the staining and the percentage of cells staining positive were determined independently by two observers without knowledge of the methylation status of the tumors. Each sample was given an intensity score (0–3) and a percentage of cells positive score (0 = 0%, 1 = 1–19%, 2 = 20–79%, 3 = 80–100%). An overall immunohistochemistry score was calculated by multiplying the intensity and percentage of cells positive scores. Statistical analysis was performed using a Mann-Whitney *U* test.

Results

Methylation Analysis of Ovarian Tumors

The methylation status of ovarian tumors was determined at ten loci using MSP. The loci chosen had either been previously reported to be methylated in ovarian cancer (*BRCA1*, *HIC1*, *MLH1*, *p16* and *hTR*) or in other tumor types (*CASP8*, *MINT25*, *MINT31*, *p15*, and *p73*). DNA from 93 ovarian tumors was modified using sodium bisulfite, which converts all unmethylated cytosine residues to uracil but leaves methylated cytosines unchanged. To confirm that the modification had been successful, all samples were first amplified with primers specific for unmethylated DNA at the *p16* or *MLH1* loci (even tumors methylated at these loci would be expected to be positive due to contamination with normal tissue which is known to be unmethylated). All tumors successfully amplified with these primers (examples in Figure 1, A and B), whereas unmodified tumor DNA did not amplify (data not shown). The samples were next subjected to MSP using primers specific for methylated DNA at the ten loci being studied.

Figure 1. Examples of methylation analysis using MSP. The methylation status of the ovarian tumors was determined by MSP at the loci indicated in the figure. For *MLH1* and *p16* (**A** and **B**), amplification was performed with primers specific for unmethylated (U) and methylated DNA (M). For all other loci (**C-H**) amplification was solely with methylated DNA-specific primers. The sample being analyzed is indicated above each lane. In all cases, *in vitro* methylated DNA (IVM) was used as a positive control for amplification with methylated DNA-specific primers.

MLH1, *HIC1*, *hTR*, *BRCA1*, *p73*, *MINT25*, and *MINT31* all showed methylation in a significant proportion of the samples (10%, 16%, 24%, 13%, 10%, 16%, and 54%, respectively; examples in Figures 1 and 2). However *CASP8*, *p15*, and *p16* were rarely if ever methylated (3%, 1%, and 0%, respectively; Figure 1 and Table 1). The majority of the samples showed methylation of at least one locus (71%) and methylation of between two and six loci in the same tumor was also frequently seen (43%; Figure 2).

Normal ovarian tissue could not be obtained to determine whether any of the loci exhibited methylation in non-neoplastic cells. However, for 18 of the patients, grossly normal tissue taken from immediately adjacent to the tumor was also analyzed. For seven of the loci (*CASP8*, *MINT25*, *MLH1*, *p15*, *p16*, *p73*, and *hTR*), no methylation was detected in any of the samples. In a small number of cases a positive signal was obtained for *BRCA1* (1/18), *HIC1* (2/18), and *MINT31* (5/18) in the normal samples. However, in all cases the signal obtained in the normal tissue was faint, suggesting that only a small fraction of the sample contained methylated DNA. This most likely represents a small number of infiltrating tumor cells or preneoplastic cells present in the normal sample, and suggests that the ten loci studied here are methylated in a tumor-specific fashion. However, low levels of methylation in normal tissue cannot be ruled out.

Analysis of Methylation Distribution

To determine whether ovarian cancer displayed evidence of coordination of methylation at multiple loci (ie, a CIMP phenotype) the Mann-Whitney *U* test was used to compare the frequency with which other loci were methylated when a particular loci was either methylated or unmethylated. The five known relapse samples were excluded from the analysis. This demonstrated that the observed methylation was distributed in a non-random fashion. In particular, four of the loci (*HIC1*, *MINT25*, *MINT31*, and *p73*) exhibited a statistically significant association with tumors exhibiting methylation of at least one other locus (Table 1). In addition, methylation at both *MLH1* and *hTR* also exhibited an increased association with methylation at the other loci, but the levels did not reach significance (Table 1). On the contrary, methylation of *BRCA1* was negatively associated with methylation of most of the other genes (*hMLH1*, *HIC1*, *hTR*, *MINT25*, and *p73*; $P = 0.02$). Overall, the results demonstrate that methylation is not randomly distributed and that ovarian cancer can exhibit the CIMP phenotype. However, not all loci were found to be coordinately methylated, suggesting the presence of at least two subgroups of CIMP-positive tumors, one susceptible to methylation of *BRCA1* (and probably *MINT31*), and another susceptible to methylation of *HIC1*, *MINT25*, *MINT31*, *p73*, and probably *MLH1* and *hTR*. In addition, 29% of the tumors were unmethylated at any of the loci tested.

There was no apparent association between the CIMP phenotypes and either grade or histological subtype of the tumors (Figure 2). Methylation at the individual loci also showed no correlation with the exception of *hTR*,

Sample	MLH1	hTR	BRCA1	HIC1	MINT25	MINT31	p73	CASP8	subtype	grade
O74									clear cell	3
O1									NS	3
O2									NS	3
O44									serous	3
O55									endometriod	3
O3									clear cell	NS
O7									-	-
O24									clear cell	NS
O26									-	-
O27									endometriod	NS
O34									serous	NS
O42									NS	2
O53									serous	3
O60									serous	3
O63									clear cell	2
O78									serous	3
O6									-	-
O10									NS	2
O15									-	-
O19									mixed	NS
O20									-	-
O21									-	-
O23									-	-
O28									NS	3
O36									mixed	3
O39									mucinous	3
O41									mixed	3
O43									serous	3
O47									mucinous	1
O51									endometriod	2
O52									-	-
O57									mixed	3
O66									endometriod	2
O69									mucinous	1
O101									serous	3
O4									-	-
O13									-	-
O22									-	-
O25									mucinous	NS
O31									serous	2/3
O35									mixed	3
O37									serous	2
O40									serous	3
O46									NS	NS
O48									serous	3
O49									serous	2
O50									NS	NS
O54									serous	3
O58									NS	3
O64									serous	3
O67									serous	3
O71									serous	2/3
O77									serous	3
O86									endometriod	3
O88									serous	3
O91									endometriod	3
O92									serous	NS
O93									serous	3
O94									serous	3
O96									serous	3
O97									NS	NS
O9									mucinous	1
O11									endometriod	3
O12									-	-
O14									-	-
O16									-	-
O17									serous	NS
O18									-	-
O29									serous	3
O30									endometriod	3
O32									serous	3
O33									serous	3
O38									mixed	3
O45									mucinous	2
O56									serous	3
O59									-	-
O62									NS	3
O65									endometriod	1
O70									NS	3
O72									mixed	NS
O73									mucinous	3
O75									serous	NS
O82									mucinous	NS
O83									endometriod	3
O87									endometriod	3
O89									mixed	3
O90									mucinous	2
O95									mixed	NS
OR8									granulosa cell	NS
OR5									NS	3
OR99*									serous	3
OR98									NS	3
OR100									NS	2

Figure 2. Methylation status of ovarian tumors. The methylation status of the 93 ovarian tumors is shown (sample designation on the left), where a **filled box** indicates the presence of methylation and an **open box** the absence of methylation. The individual loci are indicated above each column. The tumor subtype and grade are also shown where known. Known relapse samples are indicated by the designation OR and are listed separately from the other tumor samples. NS, not stated in pathology report. *O99 was also methylated at the *p15* loci.

Table 1. Summary of MSP Results

Gene	Tumors methylated (n = 88*)	Concordant	P value†
<i>MLH1</i>	6	+/-	0.34
<i>BRCA1</i>	11	-	0.02‡
<i>hTR</i>	20	+/-	0.13
<i>HIC1</i>	14	+	<0.001
<i>MINT25</i>	13	+	0.0016
<i>MINT31</i>	46	+	0.0012
<i>p73</i>	7	+	0.0027
<i>CASP8</i>	1	n/a	0.89
<i>p15</i>	0	n/a	n/a
<i>p16</i>	0	n/a	n/a

n/a, not applicable.

*Excluding 5 known relapse samples.

†For concordance with methylation at any other locus.

‡For negative concordance with *MLH1*, *hTR*, *HIC1*, *MINT25*, and *p73* methylation.

which was associated with clear cell tumors (4/4 methylated; $P = 0.004$, Fisher's exact test).

Immunohistochemical Analysis

To determine whether the methylation observed in this study was associated with a loss of expression of the corresponding gene product, we performed immunohistochemistry to assess *MLH1* and *BRCA1* expression. For quantitation of immunohistochemistry, an immunohistochemistry score was determined based on the percentage of cells positive and the intensity of the staining (described in Materials and Methods). Sixty-two of the tumors were assessed for *MLH1* expression. Although the majority of tumors exhibiting *MLH1* methylation still expressed at least low levels of the protein, there was a clear association between methylation and reduced *MLH1* expression (Table 2; $P = 0.0047$). A subset of the tumors, including most of those that exhibited *BRCA1* hypermethylation, were also assessed for *BRCA1* expression. Although complete loss of expression was only seen in tumors exhibiting methylation of the gene (represented by two tumors), overall there was not a significant difference in *BRCA1* expression between the methylated and unmethylated tumors (Table 2).

Discussion

The results presented here clearly demonstrate that hypermethylation of CpG islands is a frequent event in ovarian cancers. Seven of the ten loci examined in this study demonstrated significant levels of hypermethylation,

with frequencies ranging between 10% and 54%. Furthermore, the methylation was distributed in a non-random fashion, indicating that a proportion of ovarian cancers, as has been shown for colon and gastric cancer,^{11,12} exhibit the CIMP phenotype. However, the results also suggest that ovarian cancer has a somewhat more complex pattern of methylation, since not all genes that exhibit hypermethylation appear commonly methylated in the same tumor. *BRCA1* methylation was inversely correlated with tumors exhibiting methylation of *MLH1*, *HIC1*, *hTR*, *p73* or *MINT25*. Therefore ovarian cancer displays at least one group of CIMP-positive tumors, susceptible to methylation of, *HIC1*, *MINT25*, *MINT31*, *p73* (and possibly *MLH1* and *hTR*), and potentially a second group of CIMP-positive tumors susceptible to methylation of *BRCA1*. Alternatively, the methylation observed at *BRCA1* may be locus specific and independent of a general methylator phenotype. Further analysis will be required to determine whether other loci are coordinately methylated specifically with *BRCA1*. In addition roughly one quarter of the tumors do not show any hypermethylation. It may be that these tumors are not susceptible to hypermethylation or are methylated at loci other than those studied in this report.

The loci analyzed in this report (except *MINT31*) were all found to be methylated in <25% of the tumors, and, although the majority of tumors exhibit methylation of at least one locus, the number of tumors with three or more methylated loci is comparatively low (20%). This may indicate that a true methylator phenotype is comparatively rare in ovarian cancer. However, this analysis only included a fraction of the total CpG islands in the human genome (about 60% of human genes are associated with CpG islands²⁵), and it seems likely, therefore, that a much higher percentage of tumors would exhibit methylation of multiple loci if a greater number of CpG islands were assessed.

The cancer-specific nature of hypermethylation of many genes, in addition to the high percentage of ovarian tumors exhibiting methylation reported here and in other cancers,^{11,12,14} suggests that methylation could be a useful diagnostic marker. This is especially so since the recent demonstration that methylation of tumor DNA can be detected in the serum of cancer patients.^{26,27} Furthermore, this report suggests there may be several groups of tumors with different CIMP phenotypes. Because different genes appear to be targeted in these groups, it seems likely they will behave in biologically distinct fashions. However, further studies will be required to determine more clearly whether classifying tumors into different CIMP types will be of prognostic value. To this end, we are currently extending these studies by collecting clinical data on the patients in this study in a prospective manner and by assessing additional tumor samples.

Many reports have demonstrated that DNA hypermethylation is associated with loss of gene transcription both *in vitro* and *in vivo*.² The results for *MLH1* expression presented here are consistent with these findings, in that hypermethylation was associated with a clear reduction in *MLH1* expression. In this report we have not addressed the relationship of *MLH1* hypermethylation with microsat-

Table 2. Immunohistochemistry Scores

Gene	Methylated*	Unmethylated*	P value
<i>MLH1</i>	3.1 (7 [†])	6.4 (55)	0.0047
<i>BRCA1</i>	4.6 (10)	5.0 (14)	n/s

n/s, not significant.

*Average immunohistochemistry score.

†Number of samples analyzed.

ellite instability (MSI); however, a correlation between *MLH1* hypermethylation, loss of expression, and MSI has been demonstrated in colorectal, gastric, and endometrial cancers.^{6–8,28} In contrast, the immunohistochemical analysis of BRCA1 expression showed no correlation between methylation and reduced expression. Although there have been several previous reports of BRCA1 methylation in breast and ovarian cancer, only one has reported an association with loss of expression in breast cancer.²⁹ The results presented here suggest that BRCA1 methylation, at least in the region assessed in this report, may not play a significant role in determining BRCA1 expression levels in ovarian tumors. Recent reports using large scale analysis of CpG islands suggest that in many tumor types, up to several thousand CpG islands may exhibit hypermethylation in a single tumor.^{30,31} Clearly, only a small percentage of these methylation events is likely to be crucial in the carcinogenic process, emphasizing the need to correlate such changes in methylation with changes in expression before the significance of the observed methylation can be determined.

The results presented here show that the clear majority of ovarian tumors (71%) exhibit hypermethylation of at least one locus of the ten examined. Since, as discussed above, this represents only a tiny fraction of the potential targets for methylation, it seems likely that most ovarian tumors will have many genes targeted by methylation, a proportion of which will likely be important in the development of the tumor. As these genes are not normally methylated in adult tissue,¹ this represents one of the most prevalent tumor-specific markers yet identified. In addition, numerous studies have demonstrated that reversal of CpG island methylation can result in reactivation of the associated gene.² Consequently, reversal of promoter hypermethylation and resultant re-expression of tumor suppressor genes represents a very promising molecular target for developing novel therapies. We have previously shown that treatment of cell lines²¹ and mouse xenografts,³² which exhibit *MLH1* hypermethylation, with the DNA methyltransferase inhibitor 2'-deoxy-5-azacytidine results in re-expression of *MLH1* and resensitization to chemotherapeutic drugs. However, the use of the currently available DNA methyltransferase inhibitors is likely to be limited by their cytotoxicity.³³ Therefore, the development of a less toxic or nontoxic compound that can reverse methylation-induced transcriptional silencing will be a key step toward making widespread use of such agents feasible in a clinical setting.

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